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Efficacy of Beauveria bassiana infection and subsequent oral treatment of ehtanolic plant extract on total haemocyte count and differential haemocyte count on 3rd day of fifth instar larvae of PM and CSR2 Bombyx mori L.

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Abstract— **The present investigation shows inoculation of Beauveria bassiana causes change in Total haemocyte count (THC) and Differential haemocyte count (DHC). Subsequent treatment of plant extract Curcuma longa, Aegemone mexicana and Clerodendrum multiflorum showed the antifungal effect and maintain the normal physiological condition of B. bassiana treated larvae. After the treatment of plant extract the physiological condition is more or less similar to the control of both PM and CSR2 race of Bombyx mori L. The 25.78% and 22.09% THC was increased in B. bassiana inoculated larvae of both PM and CSR2 respectively**

Index Terms— **Beauveria bassiana, Bombyx mori, Plant extracts**.

I. INTRODUCTION

In insects, haemolymph is a circulatory fluid, with several functions, such as storage and transportation of nutrients and plays an important role in excretion, defense, moulting and metamorphosis (Mullins, 1985). The haemocytes, which actively involved in the defense mechanism by which various microorganisms from the haemocoel are removed. The cellular defenses refer to haemocyte mediated response such as phagocytosis, nodulation and encapsulation (Schmidt *et al.,* 2001).

The free haemocytes in the haemolymph of insects are responsible for the defense reaction against foreign agents that invade the haemocoel (Tepass *et al.,* 1994; Falleiros and Gregorio, 1995; Inoue *et al.,* 2001). Haemocyte present variable morphology and functions (Gupta, 1985; Ratcliffe *et al.,* 1985). The free haemocyte of lepidopterous insects have been studied in a wide range of species (Essawy *et al.,* 1985; Saxsena *et al.,* 1988; Butt and Humber, 1989, and Andrade *et al.,* 2003).

In present study experiments were designed to study antifungal role of plant extracts against *B. bassiana* infection and the functions of haemocytes along with plant extracts treatments in the recovery of silkworm larvae is made by making total and differential haemocyte count observations. Insect haemocytes are classified as the granulocytes, prohaemocytes, plasmatocytes, spherulocytes, adipohaemocytes, coagulocytes and oenocytoides (Arnold and Hinks, 1976). Major function of the haemocytes is the encapsulation of small particles and the large foreign materials haemolymph, coagulation and storage and distribution of nutritive material.

Several studies made on fungal and viral disease of silkworm but the information regarding the exact biochemical and physiological changes occurring inside the body of silkworm, throughout the progress of disease is limited. Here investigations on the disease with a view to find out changes in haemocyte of silkworm *B. mori* infected with the fungus *B. bassiana* were made. The haemocytes in the present study were classified according to the Akai and Sato (1978, 1979) and Gupta (1979) schemes.

The changes were observed in each hametocytes during the infection of pathogen and subsequent treatment of plant of extracts of PM and CSR2 race were made.

II. MATERIAL AND METHODS

Rearing of silkworm: The silkworm larvae of the races, Pure Mysore (PM) mutivoltine and CSR2 Bivoltine were reared according to the standard method as described by Krishnaswami *et al.* (1978; 1979).

Preparation of plant extract: The shade dried selected plant material was powdered and kept in ethanol or extraction for 72 hours. After extraction ethanol was allowed to evaporate. The extract obtained was stored at 10ºC until further use (Alade and Irobi, 1993; Ahmed and Beg, 2001). Plant extract was prepared in distilled water with 6000ppm and used for the treatment against the *B. bassiana* inoculated silkworm larvae of both the races selected for the present study.

LD50 value for fungi *B. bassiana*: In *B. bassiana* infected larvae, LD50 value observed in PM race was $1x10^6$spores/ml and in CSR2 was $1x10^5$spores/ml.

Inoculation of *B. bassiana* and plant extract treatment to silkworm larvae: The larvae were divided into 8 groups including control. Each group containing 50 larvae. On the first day of fifth instar the larvae were starved for 6 hours. Each larva from respective tray was deeped individually in LC50 concentration (1x106spores/mL and 1x105spores/mL). After six hours the larvae were fed with ethanolic plant extract coated mulberry leaves for that 100 µL of 10 mg/mL solution of ethanolic plant extract. The plant

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extract was given for three days at the same time in morning only. Haemocyte count was done on 3rd day of 5th instar larvae of *B. mori*.

Haemocytes count:

Total haemocyte count and differential haemocyte count was done by using the method of Praful (1994).

For THC used the following formula,

$$\text{THC} = \frac{\text{No. of cells counted X 10}}{\text{Number of 1 sq. mm counted}}$$

For DHC Wright's stain was used dilution with 1:1 with phosphate buffer. The experiment was repeated for three times.

III. RESULTS

The results obtained on 3rd day of fifth instar larvae in *B. bassiana* inoculation and subsequent treatment of ethanolic plant extracts are showed in **Table No.1.** and different types of haemocytes which were observed during present work are showed **Plate –I**.

1. Total haemocyte count (THC):

The total haemocyte count was maximum in CSR2 race i.e. 11676.67 THC/mm^3 than PM race 9086 THC/mm^3 in control group on 3rd day of fifth instar. The inoculation of *B. bassiana* showed the increase of total haemocyte count in both races by 25.78% in PM and 22.09% in CSR2 race. The treatment of ethanolic plant extracts *C. longa* showed decreased THC by 8.07% in PM and by 0.10% in CSR2. In *A. mexicana* treated group the THC decreased in both races by 10.18% and by 16.23% in PM and CSR2 respectively over the control. The treatment of *C. multiflorum* also showed the decreased THC by 30.9% in PM race and by 9.28% in CSR2 race.

The above results indicates that the increased THC in both races on 3rd day after the *B. bassiana* inoculation. The maximum increase of THC was observed in PM race than CSR2 race. The *C. longa* treated group showed decrease in THC as compared to control group, but the decreased percentage was very less and it was the nonsignificant change. In *A. mexicana* treated group decrease of THC was observed in CSR2 race but it was less than PM race. The treatment of *C. multiflorum* showed decreased THC in both races but the maximum decrease was observed in PM race than CSR2 race.

2. Differential haemocyte count (DHC):

i. Granulocyte:

In DHC observations, the granulocyte was maximum in CSR2 i.e. 32.67% than PM race which showed the 20.18% in control groups. In *B. bassiana* inoculated group the decreased granulocyte count was observed in PM by 48.7%, while the increased percentage was observed in CSR2 by 4.07% as compared to control groups. The *C. longa* treated group showed the decreased percentage in both races by 36.9% and by 28.5% in PM and CSR2 race respectively as compared to their control groups. In *A. mexicana* treated group both races shows decreased granulocyte count by 15.95% in PM race and 58.15% CSR2 race. The application of ethanolic plant extracts of *C. multiflorum* shows the decreased count of granulocyte by 91.59% and 38.18% in PM and CSR2 races respectively.

The results obtained shows that the inoculation of *B. bassiana* responsible for decreased granulocyte count in PM and increased granulocyte count in CSR2 race as compared to control group. The treatment of ethanolic plant extracts of all groups shows the decreased granulocyte count in both races on 3rd day of inoculation. The maximum decrease was observed in PM race in *C. multiflorum* treated group.

ii. Prohaemocyte:

Maximum prohaemocyte count was observed in PM race than CSR2 in control group i.e. 30.14% and 10.33% respectively. The inoculation of *B. bassiana* shows the decreased count of prohaemocyte 23.05% and 41.9% in PM and CSR2 race respectively. The application of ethanolic *C. longa* extract shows the increased prohaemocyte in PM by 65.7% while the decrease was observed in CSR2 race by 15.7% as compared to control group. In *A. mexicana* treated group of PM race, the increased count of prohaemocyte was observed by 9.85% while the decreased count was observed in CSR2 by 12.48%. In the treatment of *C. multiflorum* plant extract the percentage of prohaemocyte was observed in PM by 36.59% and in CSR2 decrease was observed by 12.4% as compared to their respective control groups.

The above results revealed that the inoculation of *B. bassiana* causes the reduction in prohaemocyte count in both races. The decrease in percentage was observed in CSR2 race than in the PM race. The application of ethanolic plant extracts after the inoculation of *B. bassiana*. The increase in prohaemocyte percentage was observed in PM race. The maximum increase in prohaemocyte was observed in *C. longa* treated group. But the decrease in haemocyte count was observed in CSR2 race and decreased prohaemocyte count was observed in *C. longa* treated group as compared to control group.

iii. Plasmatocyte:

In control group CSR2 shows the higher percentage of plasmatocyte i.e 4.33% than PM race i.e 2.06%. in *B. bassiana* inoculated group PM race shows significant increase of the plasmatocyte by 473.3% while in CSR2 increased plasmatocyte by 69.51% as compared to control group. The treatment of ethanolic plant extract of *C. longa* shows the increased plasmatocyte count in PM race by 112.6% and decreased percentage was observed in CSR2 race by 7.62% as compared to control group. In *A. mexicana* treated group in both races shows increased plasmatocyte count by 21.84% and 7.88% in PM and CSR2 races respectively. The treatment of *C. multiflorum* also observed the plasmatocyte count by 107.7% and 61.6% in PM and CSR2 race respectively as compared to control.

The above results indicate that the increased percentage of plasmatocyte was observed in the entire group including *B. bassiana* inoculated group except the *C. longa* treated CSR2 group.

iv. Spherulocytes:

The higher spherulocyte percentage was observed in CSR2 race (15.67%) than the PM race (8.19%) in the control group. The decreased spherulocyte percentage was observed in both races after the *B. bassiana* inoculation, which was by 85.71% in PM race and by 53.4% in CSR2 race. The treatment of ethanolic extract of *C. longa* shows decreased spherulocyte by 49.4% in PM race and by 10.9% in CSR2 race. In *A. mexicana* treated group increased spherulocyte

percentage was observed in both the races by 253.6% and by 5.80% in PM and CSR2 race respectively. The treatment of *C. multiflorum* shows the increased spherulocyte in PM race and decreased in CSR2 by 84.2% and by 65.7% respectively.

The above results indicate that the decreased spherulocyte count in both races due to the inoculation of fungus *B. bassiana.* The maximum percentage decrease was observed in PM race than CSR2 race. In *C. longa* treated group decreased percentage of spherulocytes was also reported. The maximum decrease was observed in PM race than CSR2. The increased or decreased percentage of spherulocyte showed the significant level as compared to control groups.

v. Adipohaemocytes:

In control, group more adipohaemocyte percentage was observed in PM race than CSR2 which were 19.66% and by 16.67% respectively. The adipohaemocyte percentage increased in both races after the inoculation of fungus *B. bassiana* by 124.5% in PM race and 19.9% in CSR2 as compared to control group. In *C. longa* treated extract shows the decreased adipohaemocyte percentage by 83.8% in PM race and by 80.02% in CSR2 as compared to control group. The treatment of *A. mexicana* also shows decreased percentage of adipohaemocyte by 95.2% and by 72.04% in PM and CSR2 respectively. In *C. multiflorum* treated group the increased adipohaemocyte percentage was observed in both races by 12.41% and by 155. 9% in PM and CSR2 race respectively.

From above results it is clear that the adipohaemocyte percentage increased in *B. bassiana* inoculated group and *C. multiflorum* treated group in both races. While the decreased percentage of adipohaemocytes was observed in *C. longa* and *A. mexicana* treated group in both races as compared to control group on 3rd day of 5th instar. The maximum decreased adipohaemocyte percentage was observed in *A. mexicana* treated larvae of PM and maximum increase was observed *C. multiflorum* treated group of CSR2 race when compared with control groups.

vi. Coagulocyte:

In DHC the coagulocytes observed 4.67% in CSR2 race and 3.93% coagulocytes were observed in PM race. In control group CSR2 race showed maximum coagulocytes percentage than PM race. The inoculation of *B. bassiana* shows the increased percentage of coagulocytes in CSR2 race. While no change was observed in PM race. The treatment of *C. longa* showed the decreased coagulocytes percentage by 29% and increased percentage by 189.5% in PM and CSR2 respectively. In *A. mexicana* treated group in both races showed the increase in coagulocytes percentage by 327.4% and 28.47% in PM and CSR2 race respectively. The treatment of *C. multiflorum* shows the decreased coagulocytes percentage by 43.7% in PM while no change was observed in CSR2 race as compared to control group.

vii. Oenocytoid:

The maximum oenocytoid percentage was observed i.e. 16.29% in PM race and in CSR2 11.67% in control group. The reduced percentage of oenocytoid was observed in both races after the *B. bassiana* inoculation. The percent decrease was 31.79% in PM and 11.48% in CSR2. The treatment of ethanolic extract of *C. longa* showed significant decrease in oenocytoids by 53.65% and by 46.70% in PM and CSR2 respectively as compared to control. In *A. mexicana* treated group PM shows decreased oenocytoid by 91.83 % and increased oenocytoid in CSR2 by 34.54% percentage over control group. While in comparison *C. multiflorum* treatment both races shows the decreased oenocytoid count by 13.38% in PM race and by 3.77% in CSR2 race.

From above observation it is clear that the *B. bassina* inoculation causes the increase of THC in both the races. The maximum increase was observed in CSR2 race than PM. The treatment of ethanolic plant extract showed that, PM race shows increased THC in all the groups out of which maximum increase was observed *C. multiflorum* treated group. But the CSR2 race shows the decreased THC than control group found in 3rd day of 5th instar larvae. The maximum decrease was observed in *C. multiflorum* as compared to control group.

IV. DISCUSSION

The treatment of ethanolic plant extracts having the antifungal activity is suppressing the multiplication of pathogen and boosting the immunity level, which leads the similar results with control or increased than control. In normal control THC was significantly low as compare to the treated became the increase may represents the defense response of silkworm against the invading pathogen.

Therefore, the observations of the present study agreed with the observations of the earlier workers. They have reported that once the entomophages fungi penetrated the host integument and gained access to nutrient rich haemolymph. The observed data of the present study agreed with the earlier investigator that the number may increase (Balvenkatasubbiah *et al*., 2001; Al-Attar, 2010) and decrease to count foreign body when infected. Earlier workers (Horohov and Dunn, 1983) have worked out the cellular responses to infection in many insect.

The similar types of observations were also obtained when the effect systematic fungicide studied on the THC in *B. bassiana* infected silkworm. *B. mori* haemocytes, are extremely efficient removing pathgen by accomplishing a series of reactions designed as phagocytosis, nodule formation. In case of DHC, the plasmatocyte and granulocyte number was increase in all groups of *B. bassiana* group than control. The similar results obtained by Anandkumar and Micchael (2011) in the *B. thuriengesis* infected larvae of *B. mori* caused significant increased number of DHC and THC as compared to control. Wago and Ishikawa (1979) reported that an increase of granular haemocyte percentage was found in *B. mori* larvae between first and fifth instar. The increased number of granulocytes and plansmatocytes can be related with the defense mechanism in *B. mori,* as both the haemocytes functions as phagocytes.

The observations made in present study that prohaemocyte undergo division in order to increase the number of granular haemocytes and plasmatocytes, which would, takes part in cellular defense mechanism as per need. Therefore in present study inoculated larvae showed the decreased proportion of prohaemocytes and increased proportion of granulocytes and plasmatocytes.

From the above results it is clear that the plant extracts which having the antifungal activity showed more or less

similar results, sometimes improved when compared with control groups of both PM and CSR2 race of *B. mori.*

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PLATE -I

Types of haemocytes

Fig.1 ` **a.** Plasmatocyte and **b.**Prohaemocyte
Fig. 2 to 4 Spherulocytes
Fig. 5 Plasmatocyte
Fig. 6 Adipohaemocyte
Fig.7 Oenocytoid
Fig. 8 **a**. Granulocyte and **b.** Prohaemocyte
Fig. 9 **a.** Spherulocyte and **b.** Prohaemocyte
Fig. 10 Coagulocyte
Fig. 11 Granulocyte
Fig. 12 **a.** Oenocytoid

PLATE -I

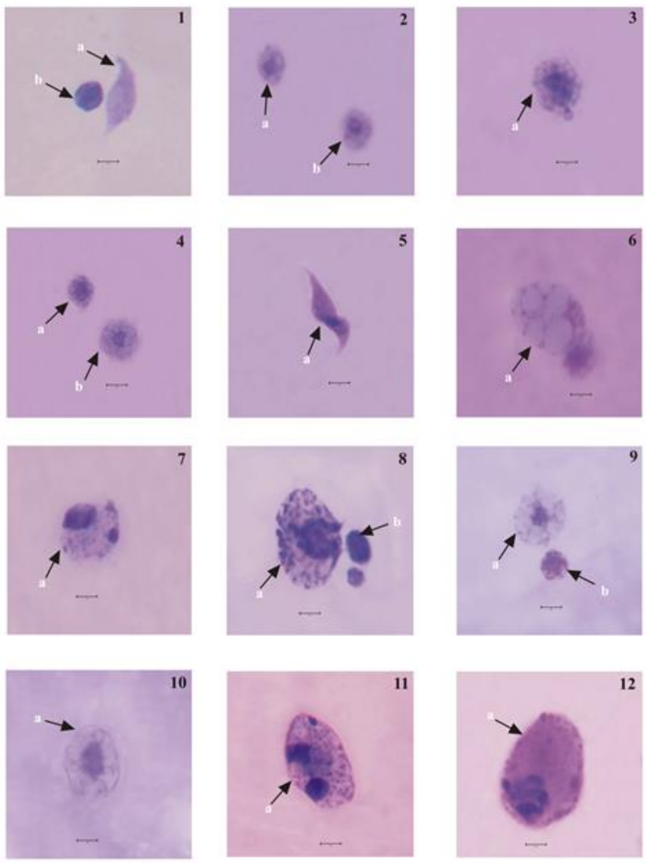

Table No. 1: Effect of *B. bassiana* infection and subsequent oral treatment of ethanolic plant extracts on total haemocyte count (THC) and differential haemocyte count (DHC) on 3rd day of fifth instar larvae of PM and CSR2 *B. mori* L

GROUPS	RACE	THC	DHC						
			GRA	PRO	PLA	SHP	ADI	COA	OEN
CONTROL	PM	9086.00 ± 166.69	20.18 ± 1.61	30.14 ± 3.14	2.06 ± 0.95	8.19 ± 0.65	19.66 ± 0.79	3.93 ± 0.12	16.29 ± 0.34
	CSR2	11676.67 ± 612.50	32.67 ± 3.79	10.33 ± 1.53	4.33 ± 3.21	15.67 ± 8.14	16.67 ± 7.02	4.67 ± 1.15	11.67 ± 4.51
INOCULATED	PM	11428.67 ± 43.39 **(+25.78)** ***	10.34 ± 0.98 **(-48.7)** ***	23.03 ± 0.03 **(-23.05)** **	11.07 ± 0.07 **(+473.3)** ***	1.17 ± 0.31 **(-85.71)** ***	44.15 ± 0.51 **(+124.5)** ***	0.00 ± (0.00)	11.11 ± 0.00 **(-31.79)** **
	CSR2	14256.67 ± 371.18 **(+22.09)** ***	34.00 ± 19.16 **(+4.07)** *	6.00 ± 4.36 **(-41.9)** **	7.34 ± 4.05 **(+69.51)** **	7.29 ± 5.03 **(-53.4)** **	20.00 ± 4.00 **(+19.9)** **	6.67 ± 0.58 **(+42.82)** **	10.33 ± 1.53 **(-11.48)** NS
C. LONGA	PM	8352.00 ± 102.53 **(-8.07)** NS	12.72 ± 1.06 **(-36.9)** **	49.96 ± 0.96 **(+65.7)** **	22.92 ± 0.66 **(+112.6)** ***	4.14 ± 0.64 **(-49.4)** **	3.17 ± 0.23 **(-83.8)** ***	2.79 ± 0.26 **(-29.00)** *	7.55 ± 0.09 **(-53.65)** ***
	CSR2	11664.00 ± 202.06 **(-0.10)** NS	23.33 ± 2.52 **(-28.5)** ***	8.70 ± 3.10 **(-15.7)** *	4.00 ± 0.61 **(-7.62)** NS	13.96 ± 5.13 **(-10.9)** **	3.33 ± 2.31 **(-80.02)** ***	13.52 ± 2.01 **(+189.5)** ***	6.22 ± 0.38 **(-46.70)** ***
A. MEXICANA	PM	8160.6 ± 115.70 **(-10.18)** **	16.96 ± 0.52 **(-15.95)** **	33.11 ± 2.11 **(+9.85)** *	2.51 ± 1.52 **(+21.84)** NS	28.96 ± 3.86 **(+253.6)** ***	0.93 ± 0.06 **(-95.2)** ***	16.80 ± 0.25 **(+327.4)** ***	1.33 ± 0.57 **(-91.83)** ***
	CSR2	9780.83 ± 296.83 **(-16.23)** **	13.67 ± 4.73 **(-58.15)** ***	9.04 ± 2.06 **(-12.48)** NS	4.67 ± 2.89 **(+7.85)** NS	16.59 ± 9.42 **(+5.80)** NS	4.66 ± 2.18 **(-72.04)** ***	6.00 ± 1.73 **(+28.47)** **	15.67 ± 3.21 **(+34.57)** NS
C. MULTIFLORUM	PM	6273.00 ± 139.19 **(-30.9)** *	1.70 ± 0.53 **(-91.59)** ***	41.17 ± 1.17 **(+36.59)** **	4.28 ± 0.60 **(+107.7)** NS	15.0 ± 1.36 **(+84.2)** ***	22.10 ± 0.20 **(+12.41)** **	2.21 ± 0.18 **(-43.7)** **	14.11± 0.30 **(-13.38)** NS
	CSR2	10592.33± 347.43 **(-9.28)** NS	20.00 ± 3.00 **(-38.18)** **	9.04 ± 2.60 **(-12.4)** NS	7.00 ± 3.61 **(+61.6)** **	5.37 ± 2.84 **(-65.7)** **	42.67 ± 4.51 **(+155.9)** ***	4.67 ± 5.51 **0** NS	11.23 ± 1.27 **(-3.77)** NS